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SINGLE-LABORATORY VALIDATION FOR THE DETERMINATION OF FREE BMAA IN CYANOBACTERIA

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EXECUTIVE SUMMARY

β-N-methylaminoalanine (BMAA) is a non-proteinogenic amino acid reported to be produced by cyanobacteria. Because of its possible links with neurodegenerative disorders such as amyotrophic lateral sclerosis and Parkinsonism-dementia complex, BMAA has generated growing interest over the past 20 years. However, its presence in cyanobacteria is still debated due to inconsistencies in the literature. As a first step towards evaluating whether BMAA occurs in New Zealand cyanobacteria, a direct quantitation method for free-BMAA was developed. The hydrophilic interaction chromatography-tandem mass spectrometry (HILIC-MS/MS) method was validated on the criteria of selectivity, linearity, sensitivity, short-term precision, intermediate precision and accuracy in two cyanobacteria samples.

BMAA was able to be measured reliably at 80 µg/kg in freeze-dried cyanobacteria. Potential interference from isobaric compounds (i.e., structurally related isomers of BMAA) was largely eliminated through chromatographic resolution and the development of highly selective mass spectrometry settings. Using the two cyanobacterial matrices available, the limit of quantitation was estimated at 100 µg/kg, but this should be evaluated further as more cyanobacterial matrices become available for analysis. As positive cyanobacterial material was not available, method precision was determined by evaluating the relative standard deviation (RSD) for replicate measurements of fortified cyanobacteria samples. The short-term precision was approximately 10% RSD_r and the intermediate precision was < 15% RSD_R. Accuracy assessments at fortification levels of 200 and 800 µg/kg were > 90% recovery. At the lower fortification level (80 µg/kg; equivalent to the lower end of the calibration curve used) recovery was lower but still > 80%.

The analytical method developed for the determination of free-BMAA in cyanobacteria was shown to be fit for use as a research tool. In the future, the method should be extended to determine the concentration of bound-BMAA in cyanobacteria samples.

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1. INTRODUCTION

β-N-methylaminoalanine (BMAA) is a non-proteinogenic amino acid that has been reported to be produced by cyanobacteria. BMAA is considered a possible cause of neurodegenerative disorders such as amyotrophic lateral sclerosis and Parkinsonism–dementia complex (ALS-PDC). The connection was first made among the Chamorro people of Guam who had extremely high rates of ALS-PDC. This was linked to the BMAA produced by *Nostoc* growing as a symbiont on cycad trees and biomagnifying through the food chain. In this instance, the Chamorro people ate fruit bats, which feed on cycad seeds which contained BMAA (Cox & Sacks 2002; Cox et al. 2003). The potential link between cyanobacteria and neurodegenerative disorders is also supported by epidemiological studies that have identified higher rates of these disorders in people living close to lakes or frequently associating with them (Sienko et al. 1990; Caller et al. 2009; Caller et al. 2012; Andrew et al. 2017; Fiore et al. 2020).

1. These observations have spurred research on the analysis of BMAA and its occurrence in cyanobacteria, microalgae and aquatic foodstuffs (Table 1). The analysis methods adopted fall into two main types:
2. Derivatisation of BMAA with reagents such as AQC (6-aminoquinolyl-N-hydroxysuccinimidylcarbamate) or chloroformate. The derivatised BMAA has increased lipophilicity, allowing it to be analysed using reversed-phase chromatography with fluorescence, ultraviolet light absorption or mass spectrometry (MS) detection.
3. Direct separation and measurement of BMAA using hydrophilic interaction chromatography (HILIC) with MS detection.

Table 1. Summary of recent analytical methodologies used to evaluate BMAA in cyanobacteria and other matrices.

Study	Methodology	Matrices Tested	Findings
Faassen et al. (2012)	AQC derivatisation with HPLC-FLD analysis [a] and direct analysis using HILIC-MS/MS.[b]	• Cyanobacteria • Cycad seed • Green algae	• Derivatisation / HPLC-FLD methods over-estimate BMAA concentrations due to low selectivity. • When direct analysis was undertaken, BMAA detected only in cycad.
Jiang et al. (2012)	AQC derivatisation with HPLC-MS/MS [c] and UHPLC-MS/MS analysis.[d]	• Cyanobacteria • Mussels • Oysters	• UHPLC and MS/MS allowed resolution of BMAA from BAMA. • BMAA detected in mussel and oyster, but not in cyanobacteria.
Jiang et al. (2013)	AQC derivatisation with UHPLC-MS/MS analysis.[e]	• Cultured cyanobacteria (*Leptolyngbya*) • Spirulina tablets (cyanobacteria-based dietary supplement)	• BMAA detected in *Leptolyngbya*, but not in spirulina (*Arthrospira* sp.).
McCarron et al. (2014)	AQC derivatisation with analysis by HPLC-MS/MS [c] or direct analysis using HILIC-MS/MS.[f]	• Cycad plant • Spirulina powder (cyanobacteria-based dietary supplement)	• BMAA detected in cycad, but not in spirulina (*Arthrospira* sp.).
Réveillon et al. (2014)	Direct analysis using SPE clean-up and HILIC-MS/MS.[b]	• Cyanobacteria • Mussels • Oysters	• BMAA detected in mussel and oyster, but not in cyanobacteria.
Beach et al. (2015)	Direct analysis using HILIC-DMS-MS/MS.[f]	• Cyanobacteria • Cycad plant • Mussels	• DMS allowed for resolution of BMAA and closely-related isomers. • BMAA detected in cycad and mussel, but not in cyanobacteria.
Réveillon et al. (2015)	Direct analysis using SPE clean-up and HILIC-MS/MS.[b]	• Cultures of cyanobacteria, green algae, dinoflagellate and diatoms • Marine plankton and periphyton • Mussels	• BMAA detected in the diatom cultures, mussels, plankton and periphyton samples, but not in the cyanobacteria or green algae or dinoflagellate samples.
Faassen et al. (2016)	Direct analysis using HILIC-MS/MS.[b]	• Cyanobacteria • Cycad seed • Seafood • BMAA-exposed *Daphnia magna* (water fleas) • Brain tissue	• SPE clean-up was required with brain tissue. • BMAA was present in the 'soluble-bound fraction'. • BMAA detected in cycad, seafood and the BMAA-exposed *Daphnia*, but not in cyanobacteria or brain tissue.

Study	Methodology	Matrices Tested	Findings
Meneely et al. (2016)	DNS derivatisation with UHPLC-MS/MS analysis.[g]	• Brain tissue	• BMAA not detected in brain tissue Alzheimer's disease patients.
Réveillon et al. (2016)	Direct analysis using SPE clean-up and HILIC-MS/MS.[b]	• Microalgae cultures • Mussels • Oysters	• BMAA detected in mussels and oysters. • Trace levels of BMAA detected in one diatom culture, but not in the majority of cultures.
Beach et al. (2018)	Direct analysis using HILIC-DMS-MS/MS.[f]	• Cycad seed • Lobster • Mussels	• Use of internal standards improved performance. • Simultaneous analysis of proteinogenic amino acids allowed hydrolysis progress to be tracked. • BMAA detected in cycad, lobster and mussels.
Foss et al. (2018)	Direct analysis using HILIC-MS/MS.[b]	• Flying fox • Mussels • Rodent species (negative control)	• BMAA detected in mussels, but not in flying fox tissue.
Violi et al. (2019)	Propyl chloroformate derivatisation with HPLC-MS/MS analysis.[h]	• Cyanobacteria	• BMAA detected in the majority of cyanobacteria cultures tested. • Strange results were observed for many cultures - free BMAA was detected in the absence of bound BMAA.
Tymm et al. (2021)	Direct analysis using HILIC-MS/MS.[b]	• Spirulina powder (cyanobacteria-based dietary supplement)	• Poor performance for total BMAA in single-laboratory validation.
Abbes et al. (2022)	Direct analysis using SPE clean-up and UHPLC-HRMS.[c]	• Lake water	• TCA addition improved detection of BMAA isomers. • BMAA was not detected in the lake water samples.

AQC = 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, DMS = Differential mobility, spectrometry, DNS = Dansyl chloride, FLD = Fluorescence detection, HPLC = High-performance liquid chromatography, HILIC = Hydrophilic-interaction chromatography, HRMS = High-resolution mass spectrometry, MS/MS = Tandem mass spectrometry, RP = Reversed-phase, TCA = Trichloroacetic acid, UHPLC = Ultra-high-performance liquid chromatography, UVD = Ultraviolet light absorption detection. [a] Nova-Pak C_{18} column. [b] SeQuant® ZIC®-HILIC column. [c] Thermo Hypersil Gold C_{18} column. [d] Agilent Bonus RP Rapid Resolution High-Throughput column. [e] Waters ACCQ-TAG™ Ultra C_{18} column. [f] TSK-gel Amide-80. [g] Waters Aquity BEH-C_{18} column. [h] Kinetex® C_{18} column.

The literature suggests there are significant inconsistencies between the two analytical methodologies described above (reviewed in Bishop & Murch 2020; see Table 1). Derivatisation methods have detected higher concentrations of BMAA than more modern HILIC methods (Faassen et al. 2012). Non-specific derivatisation of similar compounds is the likely cause of these observations and potential 'false

positives'. Because of the lack of specificity observed using derivatisation methods, a 'direct separation' approach based on HILIC chromatography and tandem-MS (MS/MS) detection was adopted here to ensure that any BMAA observations in New Zealand cyanobacteria will be reliable.

Robust quantification of BMAA also requires separation from other structurally related compounds (Figure 1) that can interfere with measurements. The most commonly observed BMAA interferences are:

- 2,4-diaminobutyric acid (2,4-DAB)
- 3,4-diaminobutyric acid (3,4-DAB)
- N-(2-aminoethyl)-glycine (AEG)
- β-amino-N-methylalanine (BAMA).

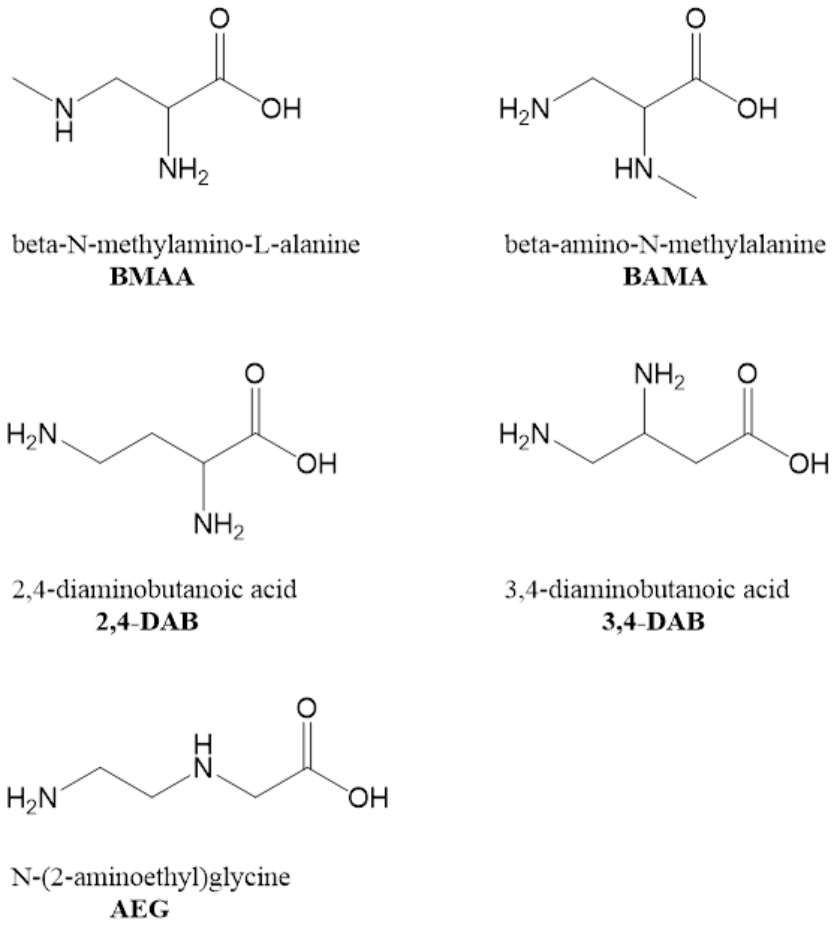

Figure 1. Structures of BMAA and structural isomers that can interfere in its analytical detection.

The key interference is likely to be from BAMA, as it appears to elute very close to or be only partly resolved from BMAA in literature studies. As the two compounds (BMAA and BAMA) share their most sensitive MS transitions, it is advantageous to obtain a good chromatographic separation between the two compounds. The use of sensitive transitions is important as BMAA has a low mass and will therefore experience relatively high noise levels from solvent impurities and low molecular weight matrix components, decreasing the sensitivity of the analysis. The other interfering isomers are not likely to cause significant issues as they are generally well chromatographically separated from BMAA. To improve accuracy (by compensating for matrix effects), a stable isotope-labelled internal standard ($^{13}C^{15}N_2$-BMAA) was incorporated.

BMAA occurs in three different forms; free, soluble-bound and insoluble-bound (Faassen et al. 2016). 'Free BMAA' is present as solely the amino acid in solution. 'Soluble-bound BMAA' is incorporated into hydrophilic proteins/peptides that are in solution. 'Insoluble-bound BMAA' is incorporated into hydrophobic proteins that are not in solution under normal conditions. Analysis of bound BMAA (both soluble and insoluble) requires the digestion of proteins/peptides to release the component amino acids (including BMAA). This project is focussed on the analysis of free BMAA, and future work will require methodology for bound BMAA to be developed in order to quantify total BMAA in cyanobacteria samples. The analytical methodology developed here (i.e., the HILIC-MS/MS method) would still be used for this, however, additional sample preparation steps would need to be incorporated compared to the analysis of free BMAA.

During the current project, a direct quantitation method for free BMAA was developed for the purpose of evaluating whether BMAA is present in New Zealand cyanobacteria. The HILIC-MS/MS method was validated on the criteria of selectivity, linearity, sensitivity, short-term precision, intermediate precision and accuracy in two cyanobacteria samples.

2. MATERIALS AND METHODS

2.1. Standards

All primary standards and reference materials were obtained from the National Research Council Canada (NRCC; Table 2). Working standards were prepared by diluting the primary standard to the required concentration using 2 mM hydrochloric acid (HCl).

Table 2. Primary standards used in this study.

Reference Material	Concentration (µg/mL)
BMAA	10
BAMA	690
2,4-DAB	650
3,4-DAB	690
AEG	960
$^{13}C^{15}N_2$-BMAA	26

2.2. Validation samples

Two samples of freeze-dried cyanobacteria were available for the validation study:

- ALG-06: Environmental benthic cyanobacterial mat material dominated by *Microcoleus autumnalis*. Collected from Te Awa Kairangi / Hutt River (Wellington Region) on 31/03/2016, freeze-dried and stored at −20 °C.
- BG-702: Cultured cyanobacterial culture of *Nostoc* sp. (CAWBG-702; Cawthron Institute Culture Collection of Microalgae). Harvested on 26/05/2022, freeze-dried and stored at −20 °C.

2.3. Sample preparation

Cyanobacteria samples were weighed into micro-centrifuge tubes and internal standard ($^{13}C^{15}N_2$-BMAA), fortification spike (BMAA) and extraction solvent (0.1 M trichloroacetic acid, TCA) were added (as per Table 3 and Table 4). Due to the limited supply of BG-702 available, the sample amount and extraction volume were reduced by 2.5-times (Table 4). Samples were extracted by sonication for 30 min and centrifuged to pellet debris (17,000 ×g for 5 min). The supernatant was transferred to a plastic autosampler vial and analysed directly by LC-MS/MS (refer to Appendix 1 for instrument parameters).

Table 3. Volumes and concentrations of internal and fortification standards added to ALG-06 samples.

Fortification Level (µg/kg)	Sample Weight (mg)	0.1 M TCA (µL)	Spike Added (µL)	Spike Stock Conc. (ng/mL)	Internal Std (µL of 1 µg/mL)
0	25	980	0	NA	20
80	25	960	20	100	20
200	25	960	20	250	20
800	25	960	20	1,000	20

Table 4. Volumes and concentrations of internal and fortification standards added to BG-702 samples.

Fortification Level (µg/kg)	Sample Weight (mg)	0.1 M TCA (µL)	Spike Added (µL)	Spike Stock Conc. (ng/mL)	Internal Std (µL of 1 µg/mL)
0	10	392	0	NA	8
80	10	384	8	100	8
200	10	384	8	250	8
800	10	384	8	1,000	8

3. VALIDATION OUTLINE

To assess the performance of the BMAA method it was tested for selectivity, linearity, sensitivity, accuracy, short-term precision and intermediate precision as described below.

3.1. Selectivity

- Differentiation of BMAA, $^{13}C^{15}N_2$-BMAA (internal standard) and interfering BMAA isomers was accomplished through chromatographic and mass spectrometric methods.
- Retention times and confirmation ratios of standards and spiked samples were compared to establish specificity.

3.2. Linearity

- A dilution series of a BMAA standard was prepared to establish the linear range for the LC-MS/MS method and was adjusted to incorporate an internal standard into the methodology.

3.3. Sensitivity

- The limit of detection (LoD) and limit of quantitation (LoQ) for freeze-dried cyanobacteria samples were determined from twenty repeat analyses of samples spiked with BMAA at a low level.
- The LoD was calculated as 2.54-times the standard deviation of the low-level repeat analyses and the LoQ was calculated as 10-times the standard deviation.

3.4. Precision

- Short-term and intermediate precision was evaluated in four replicates of cyanobacteria samples fortified with 200 µg/kg of BMAA analysed within a single day and three repeat batches analysed over three subsequent days.
- The within-day (RSD_r) and inter-day (RSD_R) precision was approximated using calculations described by International Organization for Standardization (2019).

3.5. Accuracy

- Interference (enhancement or suppression) from non-target matrix components was controlled using a stable isotope labelled internal standard.
- Two cyanobacteria samples were spiked at 80, 200 and 800 µg/kg of BMAA and the percentage recovery was evaluated.
- Four replicates were prepared for the 200 µg/mL sample, while single samples were prepared for the other levels. This assessment was repeated over four days.

4. RESULTS

An analytical method using HILIC-MS/MS was developed for the direct analysis of BMAA. The initial chromatography conditions were based on previously reported methods (Beach et al. 2015; Beach et al. 2018). Using these conditions two HILIC columns were tested; a Waters Acquity BEH Amide column and a TSK-gel Amide-80 column. The Waters Acquity BEH Amide column gave well defined peaks while the TSK-gel Amide-80 column provided poor chromatography (poor peak shape and chromatographic resolution). Using the Waters Acquity BEH Amide column, chromatography parameters were explored (column temperature, mobile phase buffer composition and gradient parameters) and an optimised chromatography method was established.

4.1. Selectivity

Several isomers of BMAA can be present in samples and have the potential to interfere with BMAA analysis due to their identical mass (BAMA, 3,4-DAB, 2,4-DAB and AEG, Figure 1). Using the developed HILIC conditions, the isomers 3,4-DAB, 2,4-DAB and AEG were well resolved from BMAA (Figure 2). However, baseline separation of BAMA could not be achieved (Figure 2) even with further optimisation of the chromatography.

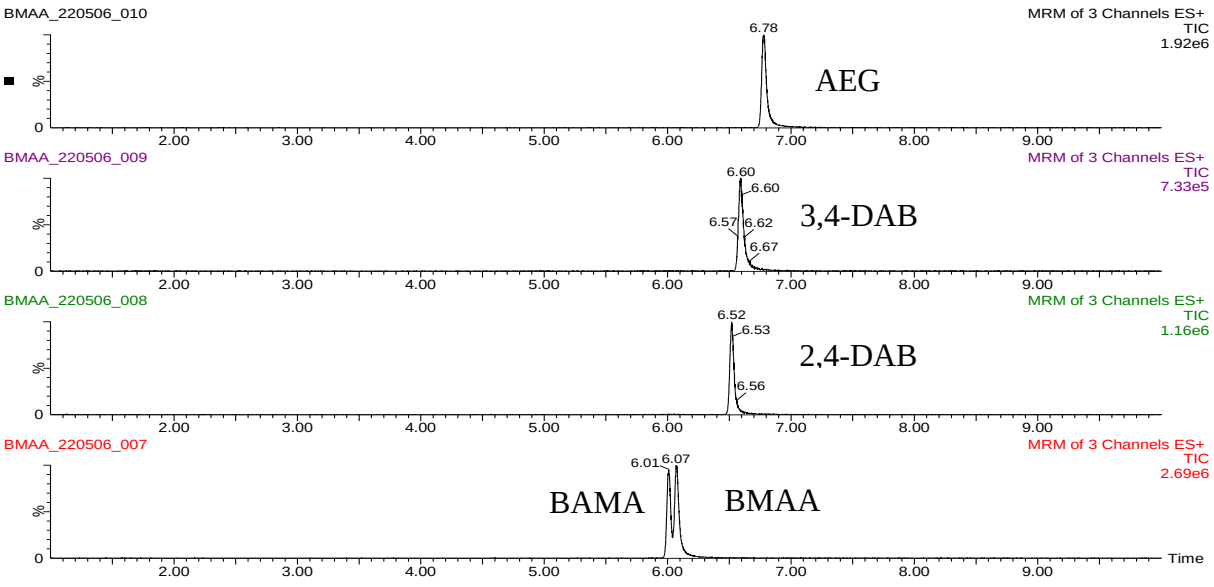

Figure 2. Example chromatograms of AEG, 3,4-DAB, 2,4-DAB and a mixture of BAMA and BMAA, showing the separation of BMAA from its isomers using the optimised HILIC conditions.

Because chromatographic resolution of BAMA and BMAA was not achievable, selective transitions for BMAA were investigated. Using the collision-induced dissociation fragmentation pathways identified from scanning experiments on BMAA (*m/z* 119.1 > 76 and *m/z* 119.1 > 44), MRM transitions were developed and collision energies were optimised. Tailoring the collision energy allowed for a high degree of selectivity between BMAA and BAMA. For the *m/z* 119.1 > 76 transition, a collision energy of 10 eV provided 500-fold higher signals for BMAA relative to BAMA (Figure 3). Using a collision energy of 5 eV provided a 200-fold higher signals for the *m/z* 119.1 > 44 transition (Figure 3). The more selective transition (*m/z* 119.1 > 76) was chosen as the quantitation channel, while *m/z* 119.1 > 44 transition was used as a confirmation channel (the qualifier).

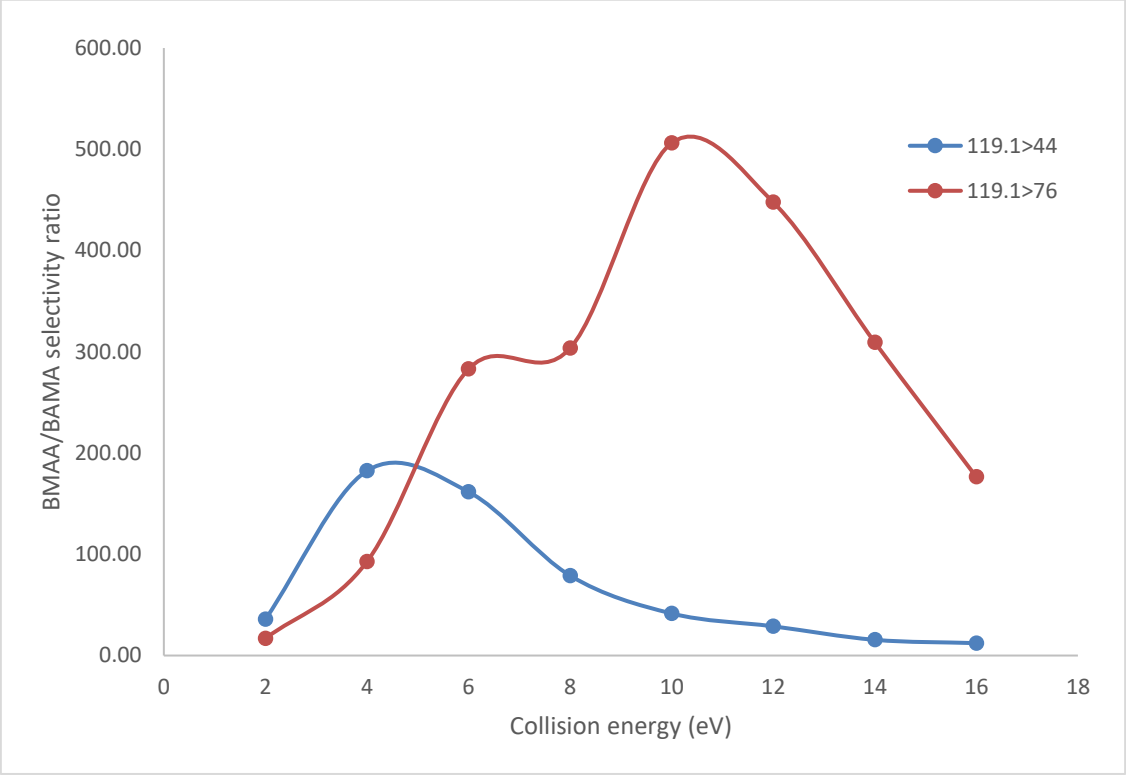

Figure 3. Selectivity of BMAA vs. BAMA for the *m/z* 119.1 > 44 (blue) and *m/z* 119.1 > 76 (red) MRM transitions when applying different collision energies.

The optimised MRM transitions for BMAA were then applied to cyanobacteria samples spiked with BMAA, internal standard and BAMA (Figure 4). Confirmation of selectivity and determining that the observed signal was not masking significant levels of isobaric compounds, was provided by comparing the ratio of quantifier/qualifier signals (Q/q) of BMAA standards to that of the spiked samples. This ratio was typically between 0.7 and 1.1, but could be more variable closer to the limit of detection. Also evident were several impurities in the BAMA transition which were not evident in the BMAA quantifier transition (*m/z* 119.1 > 102; Figure 4). Using the *m/z* 119.1 > 102

MRM transition, BMAA isomers (BAMA, 3,4-DAB, 2,4-DAB and AEG) were not observed in either of the validation samples (ALG-06 or BG-702).

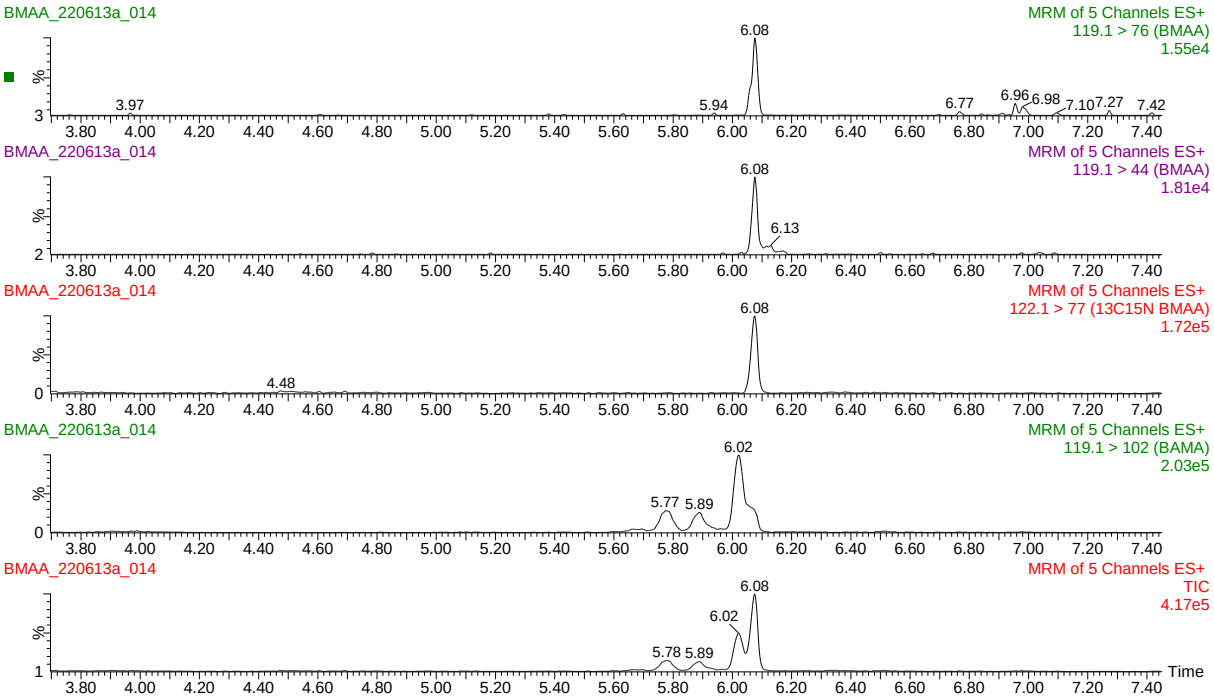

Figure 4. Example MRM chromatograms of a cyanobacteria sample spiked with BMAA, internal standard ($^{13}C^{15}N_2$-BMAA) and BAMA. Top to bottom: BMAA quantifier (*m/z* 119.1 > 76), BMAA qualifier (*m/z* 119.1 > 44), BMAA internal standard ($^{13}C^{15}N_2$-BMAA; *m/z* 122.1 > 77), BAMA (*m/z* 119.1 > 102) and a total ion chromatogram.

Selectivity for the BMAA standard and the $^{13}C^{15}N_2$-BMAA internal standard was also evaluated. The internal standard had the same peak shape as the external standard (BMAA) and near-identical retention times (Figure 5 vs Figure 6). No interference from BMAA was observed in the MRM channel for $^{13}C^{15}N_2$-BMAA (Figure 5). Similarly, no interference from $^{13}C^{15}N_2$-BMAA was observed in the BMAA quantifier or qualifier MRM channels (Figure 6).

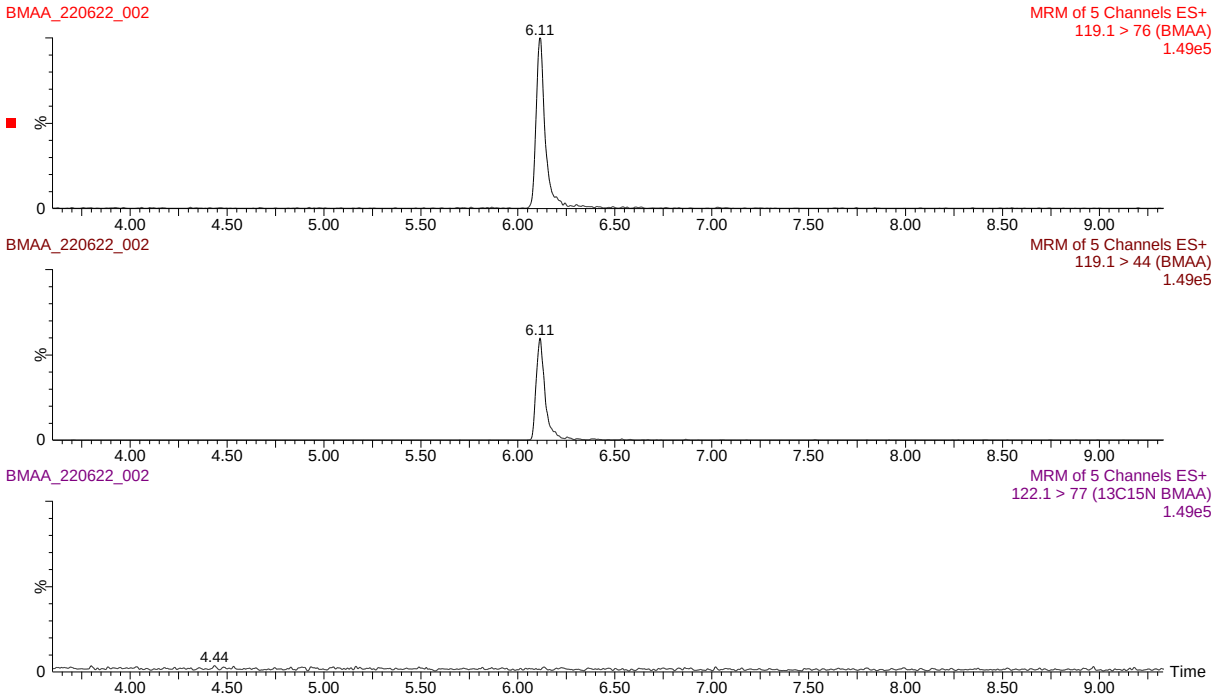

Figure 5. Example chromatograms demonstrating selectivity for the BMAA standard. Top to bottom; BMAA quantifier MRM channel (*m/z* 119.1>76), BMAA qualifier MRM channel (*m/z* 119.1 > 44) and $^{13}C^{15}N_2$-BMAA internal standard MRM channel (*m/z* 122.7 > 77).

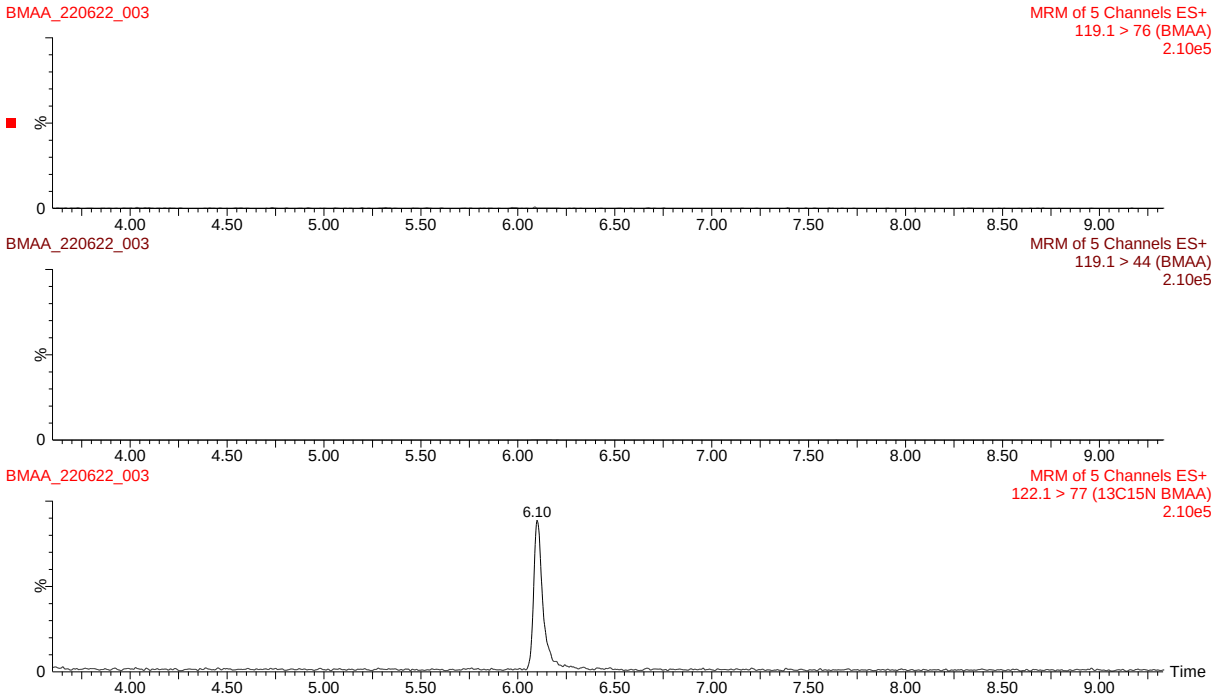

Figure 6. Example chromatograms demonstrating selectivity for the BMAA internal standard ($^{13}C^{15}N_2$-BMAA). Top to bottom; BMAA quantifier MRM channel (*m/z* 119.1 > 76), BMAA qualifier MRM channel (*m/z* 119.1 > 44) and $^{13}C^{15}N_2$-BMAA internal standard MRM channel (*m/z* 122.7 > 77).

4.2. Linearity

Initially, BMAA external standards were acquired over a range of 0.5–200 ng/mL without the use of an internal standard. This showed good linearity between 2–200 ng/mL (Figure 7), but the sensitivity below 2 ng/mL was insufficient to obtain a reliable calibration. A working calibration range of 2–100 ng/mL was chosen.

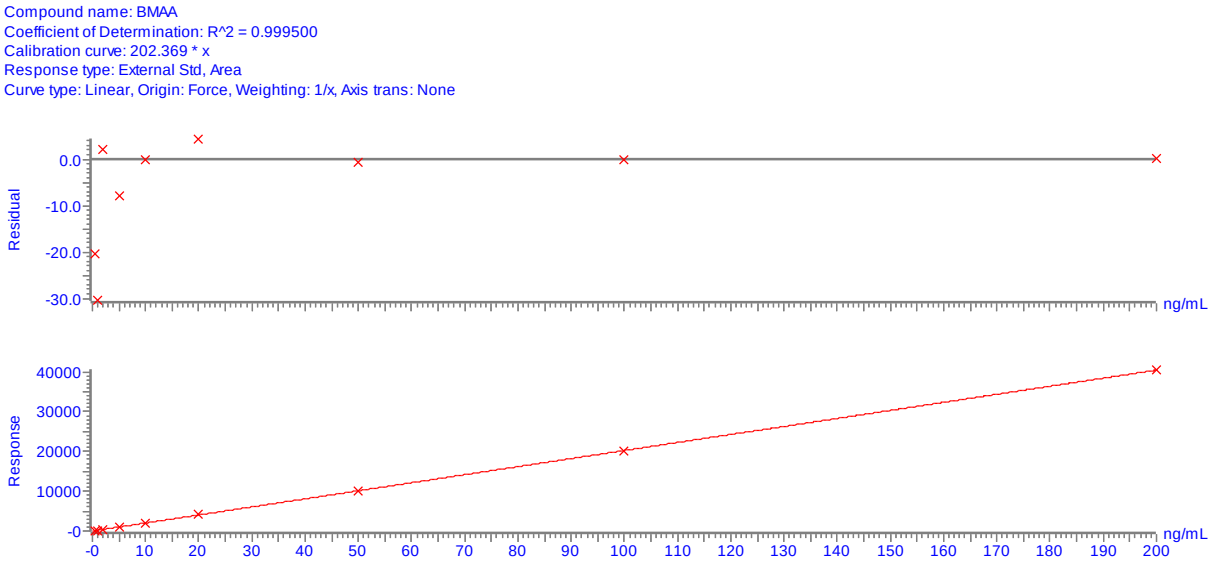

Figure 7. Calibration curve for BMAA from 0.5–200 ng/mL without an internal standard.

Following the assessment of an appropriate linear range for the external standard (BMAA), the internal standard ($^{13}C^{15}N_2$-BMAA) was incorporated to correct for matrix effects from samples. The working calibration range of 2–100 ng/mL produced a linear response for BMAA, as demonstrated by the coefficient of determination (R^2) being > 0.99. An example of the calibration is depicted in Figure 8.

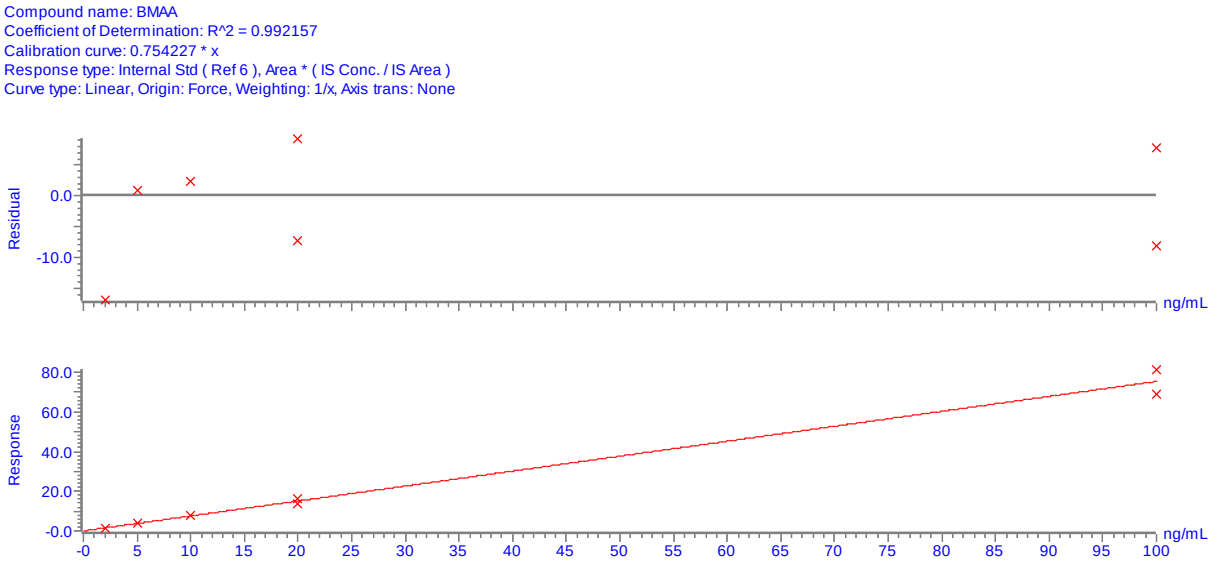

Figure 8. Typical calibration curve for BMAA using $^{13}C^{15}N_2$-BMAA as an internal standard, showing linear working range of 2–100 ng/mL.

The calibration curve was consistent over the four batches analysed during the validation study (Table 5). Using TargetLynx software, the response for $^{13}C^{15}N_2$-BMAA and R^2 for BMAA were determined. The calibration curve figure specifies the ratio of the BMAA slope vs. the $^{13}C^{15}N_2$-BMAA slope. Variation typical for LC-MS/MS was observed in the $^{13}C^{15}N_2$-BMAA slope (9% RSD). The BMAA calibration curve was highly consistent (3% RSD) due to it being relative to the internal standard.

Table 5. Calibration R^2 and response for the four validation batches.

Validation Batch	Response ($^{13}C^{15}N_2$-BMAA)	R^2 (BMAA)	Calibration Curve (BMAA/$^{13}C^{15}N_2$-BMAA)
1	102.2	0.995	0.762
2	115.9	0.998	0.732
3	95.3	0.992	0.754
4	98.0	0.995	0.715
Average	**103**	**0.995**	**0.741**
Standard Deviation	**9.2**	**0.002**	**0.02**
Relative Standard Deviation	**9%**	**0.2%**	**3%**

4.3. Limit of detection

An estimation of the theoretical LoD and LoQ was undertaken based on the procedure in Magnusson and Örnemark (2014). The procedure describes using the standard deviation of at least eight replicate sample analyses undertaken under repeatability conditions to calculate the sensitivity of an analytical method. For this study there was insufficient sample to enable this, so as an approximation was undertaken using replicate injections of a single sample preparation for each matrix.

Each cyanobacterial matrix was fortified at a level of 80 µg/kg (equivalent to 2 ng/mL, the lowest calibration level) and 20 injections were analysed for recovery. For each dataset, the standard deviation was calculated and the LoD was defined as 2.54-times the standard deviation, while the LoQ was defined as 10-times the standard deviation. From this evaluation, the theoretical LoD was 20–26 µg/kg and the theoretical LoQ was 80–100 µg/kg (Table 6).

Table 6. Theoretical limit of detection (LoD) and limit of quantitation (LoQ) calculated for the two fortified cyanobacteria samples from the validation.

Sample	SD (µg/kg)	RSD	LoD (µg/kg)	LoQ (µg/kg)
ALG-06	8	10%	20	80
BG-702	10	13%	26	100

SD = Standard deviation. RSD = Relative standard deviation. LoD = Limit of detection. LoQ = Limit of quantitation.

At the time of this validation only two different cyanobacteria samples were available in sufficient quantities for evaluation. These two matrices showed a small difference in the calculated LoD and LoQ. Therefore, the decision was made to use the higher calculated LoQ of 100 µg/kg as the limit of reporting (LoR).

4.4. Precision

Within-day repeatability (RSD_r) was generated for the two cyanobacteria samples. Four replicate samples were fortified at a level of 200 µg/kg of BMAA and within-day repeatability was calculated as per the International Organization for Standardization (2019). The short-term precision (RSD_r) was approx. 10% RSD_r for both cyanobacterial matrices (Table 7).

Between-day reproducibility (RSD_R) was generated for the two cyanobacteria samples. Each was fortified with BMAA at 200 µg/kg. Four replicates of each sample were prepared on four different days (n = 16) and between-day reproducibility was

calculated as per International Organization for Standardization (2019). The intermediate precision (RSD_R) for the ALG-06 sample was similar to its short-term precision (9.6% RSD_R), but the intermediate precision (RSD_R) for the BG-702 sample was slightly higher (13.4% RSD_R; Table 7).

Table 7. Precision data determined through the analysis of four batches of fortified cyanobacteria samples. Each batch was four replicates for each sample.

Sample	Batch	Average	SD	RSD [a]	RSD_r [b]	RSD_R [c]
ALG-06	1	5.20	0.94	18.1%	**10.6%**	**9.6%**
	2	5.09	0.49	9.6%		
	3	5.00	0.18	3.5%		
	4	5.34	0.20	3.7%		
BG-702	1	3.70	0.37	10.1%	**9.8%**	**13.4%**
	2	4.46	0.40	8.9%		
	3	4.47	0.50	11.1%		
	4	4.75	0.42	8.9%		

SD = Standard deviation. RSD = Relative standard deviation. LoD = Limit of detection. LoQ = Limit of quantitation.
[a] Within-batch repeatability determined using four replicates.
[b] Within-day repeatability (RSD_r) was calculated as per International Organization for Standardization (2019).
[c] Between-day reproducibility (RSD_R) was calculated as per International Organization for Standardization (2019).

4.5. Accuracy

No positive cyanobacteria reference material was available to evaluate the method accuracy. Therefore, accuracy was determined from fortification experiments on each of the two cyanobacteria samples. Fortification was performed at three levels (80, 200 and 800 µg/kg). Recovery in the ALG-06 sample was better than in the BG-702 sample (Table 8 and Table 9). At the 200 and 800 µg/kg fortification levels, recovery in the ALG-06 sample was 103% at both levels (Table 8). Recovery for BG-702 was slightly lower, but still around 90% (87% at 200 µg/kg and 92% at 800 µg/kg; Table 9). At the lowest fortification level (80 µg/kg; equivalent to the lowest calibration level, 2 ng/mL) recovery was lower in each cyanobacteria sample but still >80% (Table 8 and Table 9).

Table 8. Average percentage recovery for BMAA in fortified ALG-06.

Fortification Level (µg/kg)	80	200	800
Average	87%	103%	103%
Sample number (n)	4	16	4

Table 9. Average percentage recovery for BMAA in fortified BG-702.

Fortification Level (µg/kg)	80	200	800
Average	82%	87%	92%
Sample number (n)	4	16	4

5. SUMMARY OF RESULTS

The validation showed that BMAA could be reliably detected at 80 µg/kg in freeze dried cyanobacteria. At this level positives were able to be unambiguously distinguished from negatives and no false positives/negatives should occur due to the high selectivity of the method. The potential interference from isobaric compounds was largely eliminated through chromatographic resolution and by choosing highly selective mass spectrometry settings.

The LoQ / LoR was estimated to be 100 µg/kg, however, this was based on only two cyanobacterial matrices and with limited quantities of material. A formal LoD / LoQ determination will be undertaken when sufficient matrix is available and will be evaluated in a wider range of cyanobacterial matrices. This may result in the limits for both detection and quantitation being raised in the future.

Since no positive samples were available, the precision and accuracy presented in this report were generated from fortified samples at BMAA levels of 80, 200 and 800 µg/kg. Whilst the short-term precision was lower than desired (approx. 10% RSD_r), the intermediate precision was within expectations for an LC-MS/MS method (< 15% RSD_R; AOAC 2012). Intermediate precision for the BG-702 sample was lower than the ALG-06 sample. This may be due to the lower sample amount and extraction volume used for the BG-702 sample (because of a restricted amount of this cyanobacterial material being available for the validation study). Accuracy assessments at fortification levels of 200 and 800 µg/kg were acceptable (generally > 90% recovery). At the 80 µg/kg fortification level (equivalent to the lower end of the LC-MS/MS calibration, 2 ng/mL) recovery was lower but was still >80% and within the expectations provided in AOAC (2012).

In conclusion, the analytical method described here is fit for use as a research tool to determine the concentration of free BMAA in cyanobacteria. To assess total BMAA, an extension to the method will be required to determine the concentration of bound-BMAA present in cyanobacteria samples.

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8. APPENDICES

Appendix 1. Acquisition parameters for the analysis of free BMAA by HILIC-MS/MS

LC-MS/MS instrument parameters

Parameter	Description / Composition
Column	Waters Acquity UPLC BEH Amide (1.7-µm, 2.1×150 mm)
Mobile phases	A – MQ water 0.2% formic acid B – ACN 0.2% formic acid
Flow rate	0.4 mL/min
Injection volume	1 µL
Column oven	30 °C
Mass Lynx LC parameter set	BMAA_Grad.
Mass Lynx MS Tune file	BMAA.ipr
Ionisation mode	+ESI
Cone gas flow	150 L/h
Desolvation gas flow	800 L/hr
Desolvation temperature	400 °C
Source temperature	150 °C
Capillary	1.5 kV
Cone	20 V
Mass Lynx MS Experiment file	BMAA_internal_std_.MRM
Mode	MRM
MassLynx Quan method	TargetLynx (BMAA_MRM.mdb)
Quantitation mode	Area
Calibration regression	Linear, force origin, Weighting 1/X

Chromatographic mobile phase gradient

Time (min)	% A	% B	Flow rate (mL/min)	Gradient
0	10	90	0.4	Initial
10	50	50	0.4	6
15	10	90	0.4	1

Multiple-reaction monitoring acquisition parameters

Compound	Acquisition Time (min)	Parent Ion (m/z)	Daughter Ion (m/z)	Collision Energy (eV)	Dwell Time (sec)
BMAA	1-10	119.1	76.0	10	0.02
	1-10	119.1	44.0*	5	0.02
Isomers	1-10	119.1	102.0	10	0.02
$^{13}C^{15}N_2$-BMAA	1-10	122.1	77.0	10	0.02

*MRM qualifier channel.